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PRINCIPAL INVESTIGATOR: James J. Manfredi, Ph.D.

CONTRACTING ORGANIZATION: Mount Sinai Medical Center
New York, New York 10029-6574

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James J. Manfredi, Ph.D.

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Mount Sinai Medical Center
New York, New York 10029-6574

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This research application is centered on testing the hypothesis that there are mechanisms of carcinogenesis involving functional inactivation of wild-type p53 besides direct genetic alteration in human breast cancer. The goal of this proposal is to identify mechanisms and clone genes which encode proteins which act on wild-type p53 and functionally inactivate it. Technical objectives include: (1) Determine the mechanisms by which rat embryo fibroblasts acquire resistance to the growth suppressing activity of p53. (2) Elucidate the underlying mechanism for cytoplasmic localization of wild-type p53 in particular breast tumor cell lines. (3) Utilize an expression cloning strategy to screen for novel regulators of p53 function. (4) Determine the relevance of these various mechanisms in human breast cancer. The overexpression or mutational activation of such genes will then be examined in human breast tumors to determine whether the genes encoding such proteins are indeed involved in novel mechanisms of carcinogenesis. Such approaches as outlined here can address two important issues. The first is to determine whether overexpression of certain regulators of p53 may be associated with a particular prognosis or a particular success rate for a type of therapy. Second, identification of relevant regulators of p53 can allow us to begin to use such protein-protein complexes as targets for therapeutic intervention.

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James J Manfredi 8/28/98

PI - Signature Date

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Career Development Award
(08/01/97-07/31/98)

INTRODUCTION

Mutation of the tumor suppressor protein p53 which results in inactivation of p53 function is the most commonly found genetic alteration in human cancer. This research application is centered on testing the hypothesis that there are mechanisms of carcinogenesis involving functional inactivation of wild-type p53 besides direct genetic alteration in human breast cancer. This hypothesis is grounded in three observations. First, a large number of tumor viruses have been shown to express oncoproteins which inactivate the function of wild-type p53. Second, a cellular protein, mdm2, has been identified which binds wild-type p53 and inhibits its ability to act as a transcription factor. Third, mechanisms which inactivate the function of wild-type p53 have already been identified in subsets of human tumors. The goal of this proposal is to identify mechanisms and clone genes which encode proteins which act on wild-type p53 and functionally inactivate it. Technical objectives include: (1) Determine the mechanisms by which rat embryo fibroblasts acquire resistance to the growth suppressing activity of p53. (2) Elucidate the underlying mechanism for cytoplasmic localization of wild-type p53 in particular breast tumor cell lines. (3) Utilize an expression cloning strategy to screen for novel regulators of p53 function. (4) Determine the relevance of these various mechanisms in human breast cancer. The overexpression or mutational activation of such genes will then be examined in human breast tumors to determine whether the genes encoding such proteins are indeed involved in novel mechanisms of carcinogenesis. Such approaches as outlined here can address two important issues. The first is to determine whether certain types of cancer are linked with specific genetic alterations and can help to determine if overexpression of certain regulators of p53 may be associated with a particular prognosis or a particular success rate for a type of therapy. Second, identification of relevant regulators of p53 can allow us to begin to use such protein-protein complexes as targets for therapeutic intervention by designing or screening small molecular weight complexes which can disrupt or inhibit such complexes. Thus, elucidating mechanisms of carcinogenesis involving inactivation of wild-type p53 function represents an important avenue in breast cancer research.

BODY

Results

Technical Objective # 1. Determine the mechanisms by which rat embryo fibroblasts acquire resistance to the growth suppressing activity of p53. The temperature-sensitive mutant $p53^{val135}$ has been used extensively to study p53-dependent growth arrest in fibroblasts. At 37°C, $p53^{val135}$ acts like mutant p53,

whereas at 32°C $p53^{val135}$ behaves like wild-type p53 and induces growth arrest in the G1 phase of the cell cycle. Treatment of fibroblasts expressing $p53^{val135}$ with hydroxyurea or thymidine at 37°C caused arrest in the S phase of the cell cycle. Incubation of these arrested cells for as little as 4 hr at 32°C triggered apoptosis in these cells as determined by labeling with terminal transferase (TUNEL assay) and the appearance of hypodiploid cells seen after staining with propidium iodide. Similar results were seen with cells arrested in early S phase with either aphidicolin or mimosine. In contrast, cells that were arrested in early G1 with HMBA did not undergo apoptosis when shifted to 32°C. Flow cytometric analysis of the time course of asynchronous cells incubated at 32°C did not reveal any detectable apoptosis, suggesting that it is not cell cycle position which induces the apoptotic response, but actual arrest at particular points in the cell cycle. These experiments demonstrate that p53 can trigger apoptosis in S phase-arrested cells and that p53 can induce either growth arrest and apoptosis in the same cell line depending upon particular conditions.

In preliminary screening, one of the cell lines which is resistant to growth arrest (VS4-6, described in the parent grant proposal) appears to also be resistant to p53-induced apoptosis. This cell line expresses a novel p53 binding protein p140. Attempts have been made to purify enough p140 to perform microsequencing as a first step towards cloning a cDNA. These efforts will continue.

Technical Objective #2. Elucidate the underlying mechanism for cytoplasmic localization of wild-type p53 in particular breast tumor cell lines.

In addition to genetic mutation, it has been reported that nuclear exclusion of a wild-type p53 may represent a mechanism for inactivation of the tumor suppressor protein p53 in a subset of human breast tumors. To directly test whether cytoplasmic localization functionally inactivates wild-type p53, the effects of DNA damage were examined in two human breast tumor lines, MCF7 and ZR-75-1, which express a cytoplasmically localized wild-type p53. Both cell lines underwent growth arrest in response to treatment with ionizing radiation. Such treatment led to an increase in the level of p53 protein followed by an elevation in the level of the Mdm2 and p21 proteins. Interestingly, this increase in the level of p53 protein occurred in both the cytoplasm and the nucleus. Consistent with the p53 protein being transcriptionally active, luciferase reporter constructs containing p53 response elements were activated upon treatment with ultraviolet light and the p53 in nuclear extracts of UV-treated cells was capable of binding to DNA. These data suggest that in these human breast tumor cell lines, cytoplasmic wild-type p53 can function in response to DNA damage and that cytoplasmic localization *per se* does not rule out a p53-dependent response to treatment with DNA damaging agents.

Technical Objective # 3. Utilize an expression cloning strategy to screen for novel regulators of p53 function. As the research proposed for this aim is dependent on further studies under Aims #1 and 2, no progress was made on this specific aim.

Technical Objective # 4. Determine the relevance of these various mechanisms in human breast cancer. As the research proposed for this aim is dependent on Aim #3, no progress was made on this specific aim.

Discussion

The high frequency of tumor formation in the p53-knockout mice elegantly demonstrates the important role of p53 as a tumor suppressor. In contrast, the p21-knockout mice have an impaired G1 growth arrest in response to DNA damage and yet do not demonstrate a propensity to form tumors. This suggests that other functions beside DNA damage-induced growth arrest are important for the tumor suppressor activity of p53 and that p53-mediated apoptosis may be central to the tumor suppressor activity of p53. Hence our current focus on the p53-dependent apoptotic pathway in our studies of the temperature-sensitive p53 cell lines.

In regards to Technical Objective #2, we have demonstrated that two human breast carcinoma cell lines, MCF7 and ZR-75-1, which expresses a cytoplasmic wild-type p53, can respond to DNA damaging agents in a p53-dependent manner. This finding is supported by three observations. First, p53 protein levels increase and the cells undergo growth arrest after treatment with DNA damaging agents. It has been reported that upon DNA damage, cells that express a functional, wild-type p53 show an increase in the level of p53 protein and a drop in replicative DNA synthesis due to an arrest in the G1 phase of the cell cycle. It should be noted that the pattern of cell cycle distribution upon growth arrest depended on the type of DNA damage that was used. Ionizing radiation caused cells to arrest at both the G1 and G2 phases of the cell cycle, however after treatment with ultraviolet light, cells arrested in all phases of the cell cycle including the S phase. This is consistent with the findings of others demonstrating an acute cessation of replicative DNA synthesis upon treatment with ultraviolet light. Similar results were observed upon treatment with adriamycin. Second, by using biochemical fractionation, we have shown that MCF7 and ZR-75-1 cells express a cytoplasmically localized p53 and upon ultraviolet light-induced damage, the protein level of p53 increases in both the nucleus and the cytoplasm. In this assay, we have included two separate markers (lactate dehydrogenase activity and level of histone H1) to determine cross-contamination between the nuclear and cytoplasmic fractions. Two other cells lines (WI-38 and U-87 MG) express a p53 which is localized to the nucleus before and after DNA damage, suggesting that the cytoplasmic p53 that is detected in MCF7 cells is not due to a technical error in extract preparation. Moreover, the biochemical fractionation confirmed the results seen with immunocytochemical staining in MCF7 cells. Before treatment, the p53 is cytoplasmically localized and 20 hr after ultraviolet light treatment, the level as well as staining of p53 were increased in both the cytoplasm and the nucleus. It has previously been shown that treatment with DNA-damaging agents causes an accumulation of p53 in the nucleus. Few studies, however, have reported the cellular localization of p53 in untreated cells, most likely due to the inability to detect the relative low level of p53 protein that is present prior to DNA damage. Here cytoplasmic staining for p53 in untreated MCF7 cells and nuclear accumulation of p53 in response to DNA damage is detected. Third, the expression of both p21 and Mdm2 proteins was elevated following p53 induction in MCF7 and ZR-75-1 cells. The genes encoding p21 and Mdm2 are

downstream targets of p53. Induction of p21 protein has been linked to G1 arrest by modulating the activity of cyclin dependent kinases. Mdm2, on the other hand, binds to p53 and targets it for degradation suggesting that it plays a role in a feedback loop. Overall, these observations suggest that cytoplasmic localization of p53 does not prevent the cellular response to DNA damage via a p53-dependent pathway and demonstrate that a cell line that expresses cytoplasmic p53 is not resistant to DNA damage-induced growth arrest.

Adherence to Statement of Work

Technical Objective #1

During the first year, the plan was to purify sufficient quantities of p140 from the VS4-6 cell line for microsequencing and to begin to use the microsequencing data to clone the cDNA. Two attempts were made to purify p140 in large quantities. Both attempts failed to produce sufficient material for microsequencing. The protocol is currently being scaled up and a third purification will be performed.

Technical Objective #2

During the first year, the mechanism of cytoplasmic retention was to be studied and we were to begin to identify and purify the polypeptide that is responsible for the cytoplasmic anchoring mechanism. We generated convincing data demonstrating that the level of p53 is increased in response to DNA damage and that after DNA damage p53 is detectable in the nucleus. The results from these experiments suggest that cytoplasmic wild-type p53 can function in response to DNA damage and that cytoplasmic localization *per se* does not rule out a p53-dependent response to treatment with DNA damaging agents. Efforts are underway to examine in detail the mechanism of cytoplasmic retention.

CONCLUSIONS

The goal of the research contained within this proposal is to identify and clone genes which encode proteins which act on wild-type p53 and functionally inactivate it. Two potential mechanisms have been identified: the overexpression of a 140 kd p53 binding protein in the VS4-6 cell line and the cytoplasmic localization of p53 in the breast tumor cell lines MCF7 and ZR-75-1. These mechanisms are currently being examined in detail and the genes encoding the proteins that are involved will be cloned in future years. In the coming funding periods, an expression cloning strategy will be employed in an attempt to identify novel p53 regulators and the overexpression or mutational activation of the genes that have been identified by this approach will then be examined in human breast tumors to determine whether the genes encoding such proteins are indeed involved in novel mechanisms of breast carcinogenesis.